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Doctor's Dissertation

The Leucoanthocyanin from
Black Spruce Inner Bark

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THE LEUCOANTHOCYANIN FROM
BLACK SPRUCE INNER BARK

A thesis submitted by

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GLOSSARY

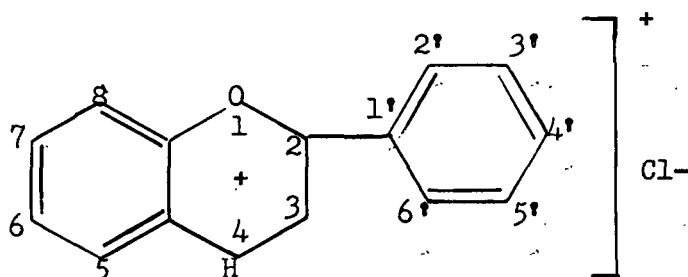
1. Paper Chromatographic Developers:

- (a) TAW, toluene-acetic acid-water (4:1:5).
- (b) BzAW, benzene-acetic acid-water (2:2:1).
- (c) EAW, ethyl acetate-acetic acid-water (9:2:2).
- (d) BAW, butanol-acetic acid-water (4:1:5).
- (e) sBW, s-butanol saturated with water.
- (f) 6% acetic acid, 6% aqueous acetic acid.
- (g) Forestall solvent, acetic acid-concentrated hydrochloric acid-water (30:10:3).
- (h) 90% formic acid-3N HCl, 90% formic acid-3N HCl (1:1).

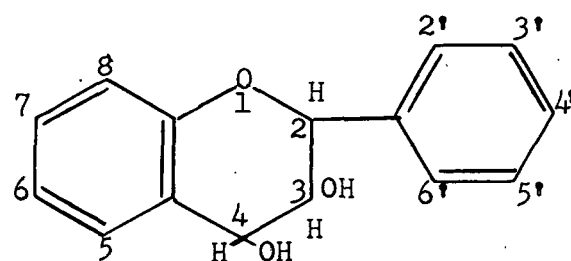
2. Spray Reagents for Paper Chromatograms:

- (a) PNA, diazotized p-nitroaniline.
- (b) Ferric chloride, 2% aqueous ferric chloride.
- (c) p-Anisidine, p-anisidine hydrochloride in butanol.
- (d) Permanganate-periodate, a mixture of four parts 2% aqueous sodium metaperiodate and one part 1% potassium permanganate in 2% aqueous sodium carbonate.

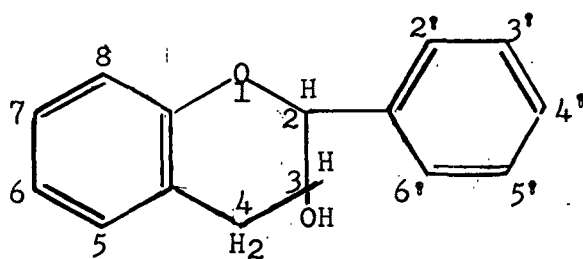
3. Basic Structural Types



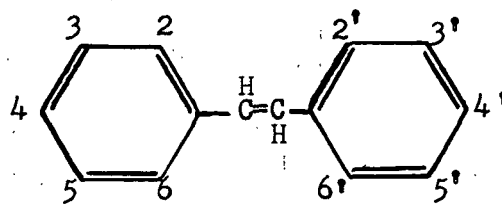
Flavylium Chloride



Flavan-3,4-diol



Flavan-3-ol
(catechin)



Trans-stilbene

INTRODUCTION

The presence of coloring matters in the wood and bark of trees has often been a determining factor in their usage. Frequently, the coloring matters were extracted from the bark and used as dyes, while the highly colored woods were used for decorative purposes. To the pulp and paper industry the coloring matters often present a serious problem, as those trees containing large amounts of coloring matters or color precursors frequently yield pulps of undesirable color even after bleaching. In order to utilize these woods more effectively, a more thorough understanding of the coloring matters and color precursors will be necessary.

Although the many classes of coloring matters and color precursors have been known for some time, many of them were poorly understood and ill defined. One such class was the leucoanthocyanins, which until recently was primarily defined by a color reaction. Modern techniques of isolation and characterization have facilitated the investigation of these compounds, as they frequently occur in small quantities and in complex mixtures which are difficult to resolve.

The primary purpose of this investigation was to isolate and characterize the leucoanthocyanin from black spruce inner bark. The ultimate goal of this work was to contribute to the better understanding of the class of compounds known as leucoanthocyanins.

HISTORICAL REVIEW

The presence in flowers and fruits of colorless materials which produce red colors upon treatment with mineral acids has long been known. These colorless materials seemed to fall into two classes, those which produced color easily and those which required heating to produce color. The former class was studied rather extensively by Willstätter and Everest (1), who determined that these materials existed as glycosides and as sugar-free materials. These compounds were red in acid solution, blue in slightly alkaline solution, and yellow in strongly alkaline solution. They are now known as anthocyanidins (sugar-free compounds) and anthocyanins (glycosides).

The second kind of colorless materials, those which required heating to produce color, gave rise to red solutions which behaved similarly to the solutions of known anthocyanidins. Rosenheim (2) named these materials leucoanthocyanins¹.

The first thorough systematic study of the leucoanthocyanins was carried out by the Robinsons (3, 4). They tested for the presence of leucoanthocyanin by treatment with hot dilute acid, and subsequently classified it according to the following solubility scheme.

- (a) Insoluble in water or the usual organic solvents or gives only colloidal solutions.

¹ The term leucoanthocyanin is used here in a general sense and does not imply the presence of a sugar group.

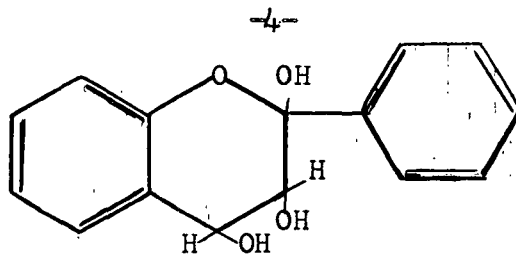
(b) Readily soluble in water, but not extracted from solution by ethyl acetate.

(c) Extractable from aqueous solution with ethyl acetate.

From this solubility scheme it is clear that the leucoanthocyanins must cover a wide range of structural types, those under type (a) probably having complex structures, with those falling into classes (b) and (c) possessing simpler structures.

The Robinsons further characterized the leucoanthocyanins by identifying the anthocyanidins produced by acid treatment through a system of qualitative color reactions and solubility tests (5). They found that a majority of the leucoanthocyanins yielded cyanidin (3,3',4',5,7-pentahydroxy flavylium chloride) and some that yielded pelargonidin (3A', 5, 7-tetrahydroxy flavylium chloride) and delphinidin (3,3',4',5', 5,7-hexahydroxy flavylium chloride) (6). They also demonstrated that the leucoanthocyanins were fairly common. Bate-Smith (7, 8) extended the work of the Robinsons using paper chromatography and found that the leucoanthocyanins were very widespread particularly in woody plants and in agreement with the Robinsons, found that cyanidin was the most commonly produced anthocyanidin.

The Robinsons (3) believed that the leucoanthocyanins were in the same state of oxidation as the anthocyanidins rather than in a reduced form as implied by their name. They proposed a 2,3,4-triol structure for the leucoanthocyanins. This structure required dehydration for the conversion to an anthocyanidin, i.e., loss of hydroxyl at position 2 or 4 and hydrogen at position 3. However, they offered no experimental proof of the proposed structure.



The Robinsons Proposed Triol Structure

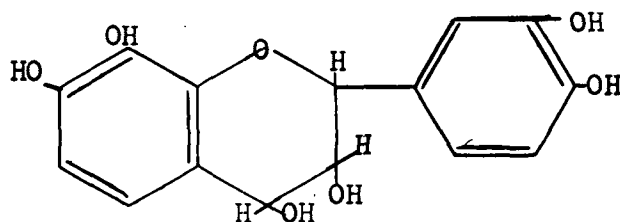
Stephens (9) offered support to the Robinsons' concept of the structure of the leucoanthocyanins. He obtained spectral evidence that the leuco-substance present in the petals of Asiatic cotton flowers (*Gossypium* spp.) was identical with the intermediate product in the reduction of quercetin to cyanidin. Since the nature of the intermediate product was uncertain, little conclusive evidence concerning the leucoanthocyanins was obtained.

In general, the isolated leucoanthocyanins have been studied by treating them with hot dilute acid and identifying the anthocyanidine produced. This method is useful, but does not establish the structure of the leucoanthocyanin. Working with an extract from cacao beans, Forsyth (10) isolated two leucoanthocyanins which yielded cyanidin. One of these materials appeared to be polymeric and was referred to as a complex leucoanthocyanin. This complex material was believed to be a glycoside, but subsequent work has shown that this compound is probably not glycosidic (11). Roux (12) chromatographed the extracts from black wattle and quebracho woods and isolated several leucoanthocyanins which yielded fisetinidin (3,3',4',7-tetrahydroxy flavylum chloride) and robinetinidin (3,3',4',5',7-pentahydroxy flavylum chloride). The differences between the leucoanthocyanins that yielded the same

anthocyanidin are not clear. They may be polymers of different molecular weight or they may be stereoisomers which are separable chromatographically.

The yield of anthocyanidin from an acid-treated leucoanthocyanin was always low (10 - 20%). Originally this fact was believed to be due to the difficulty in isolating the anthocyanidin (6). More recently chromatographic investigations of the products of an acid-treated leucoanthocyanin showed that the anthocyanidin was actually a minor reaction product (13, 14). When the reaction was run in an aqueous medium, the major product was a reddish-brown precipitate which resembled a phlobaphene. Bate-Smith and Swain (14) stated that both hydroxy catechins and leucoanthocyanins condense in the presence of aqueous acids to produce insoluble precipitates, and that the ultraviolet spectra of isolated leucoanthocyanins resembled closely those of known catechins. From these facts they concluded that leucoanthocyanins must resemble the catechins structurally. However, certain structural differences were suspected, since acid-treated leucoanthocyanins produced red solutions and red-brown precipitates, while catechins treated in the same manner yielded cream-colored precipitates and yellow solutions.

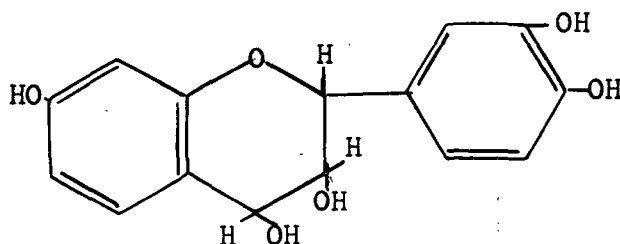
The first proof of structure for a leucoanthocyanin was established by King and Bottomley (15). They isolated from Acacia mellanoxylon an amorphous material which they named melacacidin. Degradation studies indicated a substituted flavan-3,4-diol structure, which was subsequently proved by synthesis (16, 17).



Melacacidin

When treated with hot dilute acid melacacidin yielded the corresponding anthocyanidin and a large amount of phlobaphene-like material. The authors concluded that the flavan-3,4-diol was one of the basic structural types of the leucoanthocyanins.

Support for the flavan-3,4-diol structure for the leucoanthocyanins was established by Keppler (18, 19), when he isolated a leucoanthocyanin from black wattle wood (*Acacia mollissima*) which was shown to have this structure. He named the compound mollisacacidin.



Mollisacacidin

Keppler isolated a second leucoanthocyanin which yielded the same anthocyanidin as mollisacacidin, but had a different chromatographic and spectral behavior. The structure of the second leucoanthocyanin is unknown at present.

In order to study the character of the flavan-3,4-diols and their relation to the leucoanthocyanins, a number of these compounds have been synthesized. In all cases the syntheses were carried out by reduction of a flavonol or a dehydroflavonol to the corresponding flavan-3,4-diol. Reduction of the flavonols was accomplished by catalytic hydrogenation or with lithium aluminum hydride and a catalyst (16, 17, 20). Catalytic hydrogenation is preferred as the relative spatial configuration of all substituent groups on the heterocyclic ring is more easily determined (16, 17, 21). In many cases, reduction of a flavonol with lithium aluminum hydride in the absence of a catalyst leads to the formation of the leucobase of the anthocyanidin rather than a leucoanthocyanin (22, 23).

Reduction of a dihydroflavonol can be carried out with a variety of reducing systems, including catalytic hydrogenation, lithium aluminum hydride, and sodium borohydride (24 - 27). When the reduction is carried out on a dihydroflavonol, the relative spatial position of the substituent groups on the heterocyclic ring is uncertain, since in most cases the spatial configuration of the starting material is not known (17).

The synthetic products have been useful in testing the hypothesized flavan-3,4-diol structure for the leucoanthocyanins. In several instances leucoanthocyanins which appeared to be polymeric have been isolated (3, 4, 12, 28). These complex materials yielded anthocyanins just as the monomeric compounds did. Swain (27) condensed his synthetic flavan-3,4-diol using cold dilute hydrochloric acid and obtained

a material resembling the complex leucoanthocyanins, thus demonstrating that the flavan-3,4-diols are capable of condensing to yield polymeric materials.

The flavan-3,4-diol structure is very closely related to the leucoanthocyanins, but does not fit their requirements in all cases. Joshi and Kulkarni (26) treated synthetic 6-methyl-4'-methoxy-flavan-3,4-diol with hot dilute acid and obtained a yellow-colored solution. The product was probably a flavylium salt, but due to the type of substitution it did not produce the characteristic red color. A certain type and degree of substitution appears to be necessary to produce red color upon acid treatment, probably hydroxylation in the 4' and 7 positions.

The Robinsons (3) proposed that the leucoanthocyanins were in the same state of oxidation as their anthocyanidins and that conversion required only dehydration. On the other hand, an oxidative step is necessary for the conversion of a flavan-3,4-diol to an anthocyanidin. Thus, a question is raised concerning the mechanism of conversion of leucoanthocyanins to anthocyanidins. In some cases oxygen is not required for conversion (3), while in others it appears to be essential (11). In the former case a possible mechanism is a disproportionation leading to the simultaneous formation of a flavan-3-ol (catechin) and the anthocyanidin. The presence of catechin in the reaction products was noted by Forsyth (13). On the other hand Swain (11) did not detect any catechin when he treated his synthetic flavan-3,4-diol with hot dilute acid. These facts indicate that all leucoanthocyanins may

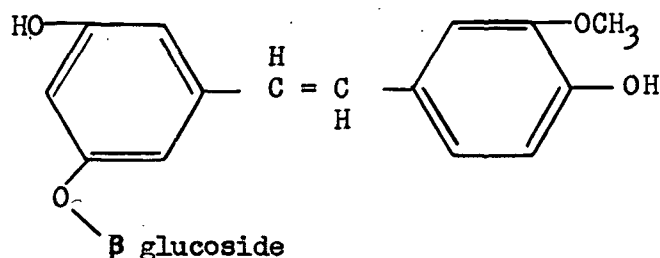
not be simple flavan-3,4-diols, and that the mechanism of conversion may not be the same for all leucoanthocyanins.

The low yields of anthocyanidin obtained from the acid-treated leucoanthocyanins mentioned previously may be attributed to a competing reaction, the acid-catalyzed condensation of the flavan-3,4-diols to phlobaphenes. Freudenberg and Weinges (29) have shown that this reaction is characteristic of the hydroxy flavans. They found that condensation is enhanced by hydroxylation at the 4' and 7-positions and that hydroxylation of the heterocyclic ring had no effect on the condensation. It is obvious that flavan-3,4-diols can undergo condensation and that this may be a partial explanation for the low yields of anthocyanidins.

Prior to the demonstration of the flavan-3,4-diol structure for certain leucoanthocyanins, Pigman and Anderson (30, 31) detected the presence of a leucoanthocyanin in black spruce inner bark. Further investigation of the inner bark lead to the isolation of a material believed to be a fairly pure leucoanthocyanin. Acetylation of this material yielded a crystalline product which was believed to be a derivative of the leucoanthocyanin. Methylation studies were also performed. However, sufficient evidence was not obtained to fix the structure of the products.

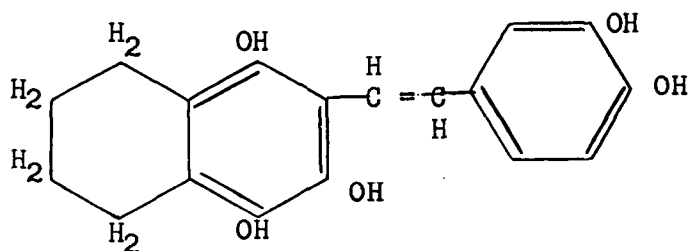
Recently, investigations of various spruce barks have lead to the isolation of two stilbene glucosides. Andrews (32) isolated a substituted stilbene glucoside from white spruce bark with properties

similar to those of the extract obtained by Pigman *et al.* (31) and which yielded a crystalline acetate similar to the acetate obtained by Pigman. Andrews (32) and Hoffman (33) proved the structure of this compound by degradation and synthesis.



Andrews' Stilbene Glucoside

Grassmann and co-workers (34, 35, 36) isolated and identified another stilbene glucoside and its aglucone from the ethyl acetate extract of spruce bark. They also isolated the dihydro-derivative of the aglucone.



Aglucone of Grassmann's Stilbene Glucoside

PRESENTATION OF THE PROBLEM

Pigman et al. (31) isolated a material from black spruce inner bark which they called the "black spruce inner bark leucoanthocyanin". Attempted characterizations through derivatives and analysis of the isolated products were inconclusive, and only few inferences could be drawn as to the structure of this "compound". The authors believed that their preparation was not completely pure, and they suggested that further work would be necessary to fully characterize the leucoanthocyanin.

Recent advances in the chemistry of the leucoanthocyanins have indicated that some of these compounds are flavan-3,4-diols, and that they exist both as simple and complex materials. In addition, Andrews (32) obtained a material from white spruce inner bark which resembled the "black spruce inner bark leucoanthocyanin" in many properties, but which was not a leucoanthocyanin.

In view of these facts and the indication by Pigman et al. that their preparation was impure, two objectives for this research were undertaken. The primary objective was to isolate the leucoanthocyanin from black spruce inner bark and determine whether it conforms with the current theories concerning the leucoanthocyanins. The secondary objective was to investigate the "black spruce inner bark leucoanthocyanin" for components other than the true leucoanthocyanin.

GENERAL EXPERIMENTAL METHODS

PAPER CHROMATOGRAPHY

Whatman No. 1 paper was used for both one-dimensional and two-dimensional paper chromatography. Chromatograms were developed by the descending method and then removed from the tanks and allowed to dry in the air. The dried chromatograms were examined under ultraviolet light and then sprayed with various chromogenic spray reagents.

The developers used for the paper chromatograms were as follows:

- (a) the upper phase of toluene-acetic acid-water (4:1:5) (TAW) (37);
- (b) the upper phase of butanol-water-17N ammonium hydroxide (50:28:4) (BAm) (38);
- (c) the upper phase of benzene-acetic acid-water (2:2:1) (BzAW) (39);
- (d) n-butanol-pyridine-saturated aqueous sodium chloride (1:1:2) ((BPS) (40);
- (e) ethyl acetate-acetic acid-water (9:2:2) ((EAW) (41);
- (f) the upper phase of n-butanol-acetic acid-water (4:1:5) (BAW) (42);
- (g) s-butanol saturated with water (sBW) (43);
- (h) 6% aqueous acetic acid;
- (i) acetic acid-concentrated hydrochloric acid-water (30:3:10) (Fore-stall solvent) (44);
- (j) 90% formic acid-3N hydrochloric acid (1:1) (45).

Two-dimensional paper chromatography was found very useful in separating the complex mixtures encountered in this work. A solution of the mixture was spotted in the corner of a 30 cm. by 30 cm. sheet of

paper and developed for four hours in 6% acetic acid. The paper strip was removed, air dried, and developed at right angles to the original solvent direction in SBW for 18 hours. The dried chromatogram was examined by the usual techniques.

The spray reagents used on the paper chromatograms included:

- (a) Diazotized p-nitroaniline (PNA) (46). Just prior to using, 0.5 ml. of 5% sodium nitrite was added to 5 ml. of a 0.5% solution of p-nitroaniline in 2N hydrochloric acid. To this solution was added 15 ml. of 20% sodium acetate. The chromatograms were sprayed with this solution and allowed to air dry. They were then sprayed with a saturated sodium carbonate solution. This spray was found to detect all phenolic materials encountered in this work excepting phenol.
- (b) 2% Ferric chloride in water (39). This spray was used to detect ortho-dihydroxyphenols and orthohydroxybenzoic acids.
- (c) p-Anisidine hydrochloride in butanol (47). Crystalline p-anisidine hydrochloride was dissolved in butanol to give a 0.5% solution. After spraying the chromatogram with this solution, it was placed in an oven at 105°C. to develop color characteristic for aldoses.
- (d) Permanganate-periodate (48). A mixture of four parts 2% aqueous sodium metaperiodate and one part 1% potassium permanganate in 2% aqueous sodium carbonate was sprayed on the chromatogram. The entire chromatogram took on a permanganate color. Gradually, yellow spots developed where the spray had reacted. The spray was used to detect sugars, glycosides, and some phenolic compounds.
- (e) n-Propanol 0.3N in hydrochloric acid. The spray was prepared by

adding 0.5 ml. of concentrated hydrochloric acid to 20 ml. of n-propanol. The chromatogram was heavily sprayed with the reagent and placed in an oven at 105°C. In about two minutes the leucoanthocyanin developed a faint but distinct pink color and d-catechin developed a bright yellow color.

COLUMN CHROMATOGRAPHY

A mixture of Magnesol - Hyflo Supercell (4:1) was used in column chromatography experiments. The adsorbent was prepared by suspending it in a methanol:water mixture (1:1) for two hours and then washing several times on a Buchner funnel with absolute methanol. The adsorbent was then slurried in absolute methanol and packed in a column. The materials to be chromatographed were dissolved in methanol and added to the column. The column was eluted with methanol and then with methanol containing approximately 1% glacial acetic acid. The eluates were filtered through a cotton plug and concentrated at reduced pressure. The concentrated solutions were then used for whatever additional studies were performed. This method is a slight modification of the technique used by Ice and Wender (49) for the separation of flavonoid materials, and the method used by Andrews (32) for the isolation of his stilbene glucoside.

DETERMINATION OF INFRARED SPECTRA

All infrared spectra determinations were made with a Perkin-Elmer Model 21 Recording Infrared Spectrophotometer by Mr. Lowell Sell of the Analytical Department of The Institute of Paper Chemistry. Samples for

spectra determination were prepared as potassium bromide pellets.

DETERMINATION OF ULTRAVIOLET SPECTRA

A Beckman Model DU Quartz Spectrophotometer was used for all ultraviolet and visible spectra determinations. The solvent used was 95% ethanol purified by refluxing with powdered zinc and potassium hydroxide and distilling from all glass equipment (50).

SOLVENTS AND REAGENTS

All solvents and reagents used were reagent grade. In some instances specially purified solvents were used. These instances have been noted in the text.

AUTHENTIC SAMPLES OF COMPOUNDS

Authentic samples of protocathechuic acid, p-hydroxybenzoic acid, vanillic acid, pyrocatechol, resorcinol, and phloroglucinol were obtained from Dr. I. A. Pearl. All other samples were obtained from commercial sources.

MICROANALYTICAL DETERMINATIONS

The microanalytical determinations of the carbon, hydrogen, and methoxyl content of the purified acetate were made by the Huffman Microanalytical Laboratories, Wheatridge, Colorado.

EXPERIMENTAL DATA

COLLECTION OF THE INNER BARK

The black spruce (Picea mariana BSP) inner bark samples were collected from trees felled in the Ripco Experimental Forest, Rhineland, Wisconsin, in June of 1957. Collection at this time insured that the bark could be easily stripped from the trees. Three trees were felled, the branches removed, and the stems cut into two-foot bolts for ease in handling. Each bolt was treated in the following manner. With the aid of a potato peeler the rough outer bark was carefully removed and discarded. The soft sticky inner bark was cut into two-inch strips and peeled from the wood bolts. The bark strips were cut into sections approximately two inches long and placed in jars containing methanol. The methanol served a twofold purpose, to arrest enzyme action and to serve as the extracting agent. Basal sections were taken from each tree and returned to the laboratory to determine basal diameter and age of the tree. The biometric data for these trees are reported in Table I.

TABLE I

BIOMETRIC DATA

Tree	Age, years	Butt Diameter, cm.	Height, feet
1	39	10.2	24
2	37	12.7	24
3	39	10.2	24

METHANOL EXTRACTION AND FRACTIONATION OF THE EXTRACT

The extraction was allowed to continue for two weeks at room temperature. At the end of this time the methanol solution containing water from the bark was filtered off, and the inner bark again covered with methanol. The second extraction was allowed to continue for two months. It was necessary to use room temperature extraction, since previous work indicated that hot extraction changed the nature of the extract (31).

The method of fractionation was a slight modification of the procedure used by Pigman et al. (31) and is depicted in Figure 1. The methanol-water solution from the first extraction was concentrated at reduced pressure until all the methanol had been removed. A brownish-colored precipitate separated as the last traces of the methanol were removed. This material was removed by filtration, dried and weighed. The aqueous solution was diluted with water and saturated with sodium chloride. This treatment produced a reddish-brown gummy precipitate. The resulting mixture was filtered over Fibra-Flo until a clear filtrate was obtained. The residue on the filter pad was extracted with methanol and the extract evaporated to dryness and weighed. The filtrate was extracted exhaustively with ethyl acetate. The resulting ethyl acetate solution was dried over sodium sulfate and sodium bicarbonate, concentrated to a small volume dried again with sodium sulfate, and taken to dryness at reduced pressure.

Fresh Inner Bark (immersed immediately in methanol)

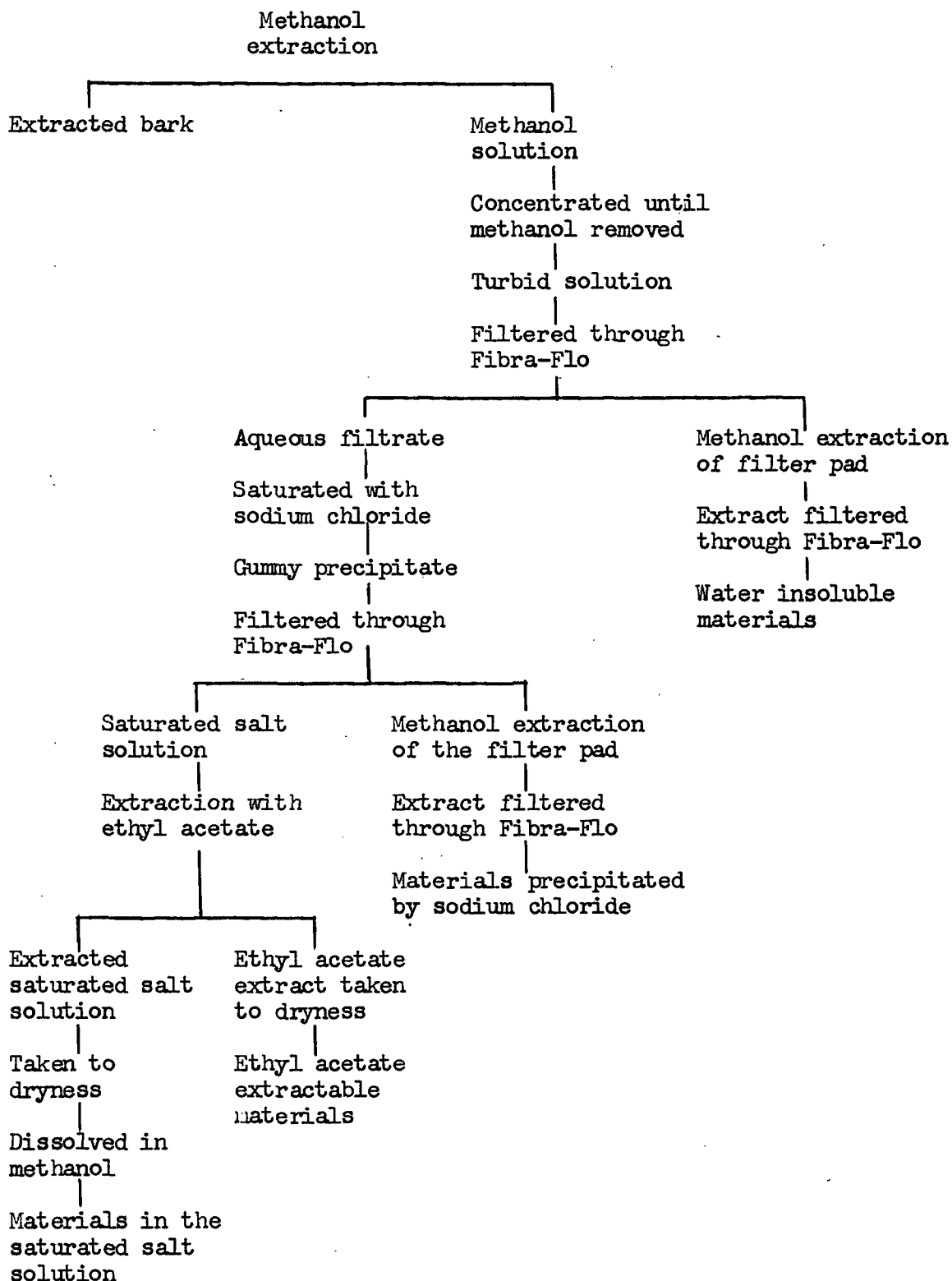


Figure 1. Preparation and Fractionation of the Extract

An aliquot of the saturated salt solution was taken to dryness and redissolved in methanol to separate the salt from the organic materials. The methanol solution was again taken to dryness and redissolved in methanol to remove the last traces of salt. This methanol solution was taken to dryness and the residue weighed. The total solids in the saturated salt solution were calculated from the solids in the aliquot.

Listed in Table II are the yields of the various fractions. Evidently the ethyl acetate fraction represents a very minor portion of the inner bark and a rather small part of the total extracted materials. Pigman *et al.* (31) designated this fraction the "black spruce inner bark leucoanthocyanin", and used it for their studies of the leucoanthocyanin. In the present work this fraction was called the "ethyl acetate fraction" and was used as the starting material for the isolation of the leucoanthocyanin.

TABLE II

YIELDS OF FRACTIONS FROM THE FIRST EXTRACT

Fraction	Yield Based on Inner Bark, % ^a	Yield Based on Total Extracted Material, %
Water-insoluble materials	1.2	7.3
Salt-precipitated materials	6.9	42.0
Ethyl acetate-extracted materials	1.6	9.6
Materials remaining in the saturated salt solution	6.8	41.0

^a Yields calculated on an oven-dry unextracted basis.

Excepting for a solids determination, the second extract was not

examined further. The yields of extracted material are given in Table III.

TABLE III

YIELDS OF MATERIALS EXTRACTED

Extract	Yield, % ^a
First extraction	16.5
Second extraction	6.1
Total	22.6

^a Calculated on the basis of the oven-dry, unextracted inner bark.

THE NATURE OF THE ETHYL ACETATE FRACTION

PAPER CHROMATOGRAPHY

Preliminary chromatographic examination of the ethyl acetate fraction showed that one-dimensional paper chromatography would not separate the components. Therefore, two-dimensional paper chromatography was adopted as a standard method of examining this fraction and its components. Development in 6% acetic acid followed by SBW was found to be the most successful system. This technique is fully described in a previous section.

A typical two-dimensional chromatogram of the ethyl acetate fraction showed 12 spots. Table IV lists the reactions of these spots with the spray reagents, their behavior under ultraviolet light, and their R_f values. The pink color developed at spot D when the chromatogram was sprayed with propanol 0.3N in hydrochloric acid and heated, marked it as

the leucoanthocyanin. A chromatogram of authentic d-catechin showed a spot corresponding to spot G in position and color reactions with the spray reagents. It was therefore concluded that spot G represented d-catechin. Spots A and B, the largest on the chromatogram, are of unknown nature. Negative results with the propanol 0.3N in hydrochloric acid spray indicated that they were not leucoanthocyanins. They did not react with the ferric chloride spray, but reacted readily with the permanganate-periodate spray. Their strong ultraviolet fluorescence indicated that they were highly conjugated aromatic compounds.

A two-dimensional chromatogram of the "black spruce inner bark leucoanthocyanin" preparation obtained by Pigman et al. (31) was identical with the chromatograms of the ethyl acetate fraction obtained in the present work.

COLUMN CHROMATOGRAPHY

A series of experiments were run with columns of unwashed Magnesol to determine optimum operating conditions. These experiments indicated that addition to the column and the first elution were best carried out with methanol. The methanol removed about 90% of the material added to the column, but did not remove the leucoanthocyanin. It was necessary to elute with methanol containing 0.5 to 1% acetic acid to remove the leucoanthocyanin. Unfortunately, this eluting agent also dissolved some of the Magnesol so that the eluate also contained dissolved inorganic material. This problem was overcome in part by dissolving the eluate in 1% aqueous acetic acid and extracting the organic materials

with ethyl acetate. Unfortunately, the extraction was incomplete. This problem limited the usefulness of a Magnesol column for isolation work.

TABLE IV

PAPER CHROMATOGRAPHY OF THE ETHYL ACETATE FRACTION

Spot	R_f 6% Acetic Acid	R_f sBW	PNA	Color Reactions				Fluores-
				Propanol- 0.3N HCl	0.2% FeCl ₃	Permanga- rate-Periodate		cence
A	0.20	0.60	DkBr	-	-	+	+	+
B	0.25	0.70	P	-	-	+		+
C	0.12	0.87	Br	-	-	-		+
D	0.45	0.55	Y-Br	Pk	+	+		none
E	0.30	0.60	Y	-	-	-		none
F	0.30	0.70	Y	-	-	-		none
G	0.37	0.87	O	Y	+	+		none
H	0.61	0.50	-	-	-	-		+
I	0.63	0.87	LtBr	-	-	-		+
J	0.70	0.90	P	-	-	-		none
K	0.80	0.75	P	-	-	-		none

Br - brown
O - orange
P - purple
Pk - pink
Lt - light color
Dk - dark color
Y - yellow

Treatment of the methanol eluate with hot dilute acid did not produce a red color. Paper chromatographic examination of this eluate showed spots A, B, C, G, H, and I. Chromatograms of the methanol-acetic

acid eluate contained spot D (the leucoanthocyanin), spot G (d-catechin), and small spots for A and B. In addition to these spots, four new spots were found which were fluorescent but did not react with any of the spray reagents. In the position occupied by spot H, two new spots were found. In the position of spot A, a third new spot was detected, and in place of spot I the fourth new spot was found. These new spots bring the total number of constituents in the ethyl acetate fraction to fifteen.

In an effort to obtain a pure preparation of the leucoanthocyanin, extrusion of the Magnesol column was tried. The column was prepared and eluted with methanol in the usual manner; then methanol containing 1% acetic acid was added until several fluorescent bands appeared to be separated. The column was then extruded and cut into zones according to the fluorescent bands. Each section was analyzed by paper chromatography. The chromatograms showed that the leucoanthocyanin was still quite impure and that no pure compounds had been separated. Therefore, this method was discontinued.

Since unwashed Magnesol was unsatisfactory, other adsorbents were tried. Acid-washed Magnesol and bicarbonate-washed Magnesol would not separate the leucoanthocyanin from the bulk of the other components in the ethyl acetate fraction. When alumina was tried, the leucoanthocyanin could not be recovered from the column. In view of these results column chromatography was abandoned as a means of isolating the leucoanthocyanin.

The results of the work with column chromatography demonstrated that 90% of the ethyl acetate fraction was not leucoanthocyanin,¹¹ and that the leucoanthocyanin probably represented less than 10% of this fraction. Furthermore, it seemed likely that the constituents represented by spots A and B represented a large portion of the ethyl acetate fraction.

AQUEOUS ACID HYDROLYSIS

Pigman et al. (31) reported that on hydrolysis the ethyl acetate fraction yielded 40% reducing sugar. They demonstrated that the sugar was glucose by forming the phenylosazone and comparing it with authentic glucosazone. To verify these results, small samples of the ethyl acetate fraction, the methanol eluate from the Magnésol column, and Pigman's ethyl acetate fraction were hydrolyzed and analyzed for glucose. The samples (5 mg.) were sealed in small glass ampoules containing 1 ml. of 2% hydrochloric acid and were subsequently heated in a steam bath for one-half hour. The hydrolyzates were analyzed for glucose by paper chromatography using EAW as the developer and p-anisidine as the spray reagent. The amount of glucose in the hydrolyzate was estimated visually by comparing the area and depth of color of the glucose spots from the hydrolyzate with spots of known glucose concentration run alongside. This method of estimation is probably accurate to $\pm 5\%$ (51, 52).

Both the ethyl acetate fraction isolated by Pigman and the one obtained in this work yielded 20 \pm 5% glucose, while the methanol eluate

yielded $25 \pm 5\%$ glucose. The 20% yield of glucose is one-half of that reported previously. The difference may be attributed to the method of analysis. Pigman used the Munson-Walker method of estimating reducing sugars, and this method is sensitive to all reducing materials. Small amounts of noncarbohydrate reducing materials present in the hydrolyzate were probably responsible for the high result.

A hydrolysis was run on the methanol-acetic acid eluate from the Magnesol column. Small amounts of glucose were detected, but quantitative results could not be obtained because of the Magnesol in the sample.

Two-dimensional paper chromatograms of the hydrolyzates showed that all the phenolic materials in the ethyl acetate fraction were affected by acid. Two prominent new spots were detected in the hydrolyzate of the methanol eluate. These spots did not move in 6% acetic acid, but moved very rapidly in the SBW developer. These spots corresponded in size to spots A and B on the original chromatogram. It seemed probable that the major components of the ethyl acetate fraction, those represented by spots A and B, were glucosides and that these two new spots represented the aglucones of these compounds.

COUNTERCURRENT DISTRIBUTION

Since column chromatography did not lead to the isolation of the leucoanthocyanin, another method of isolation was sought. Countercurrent distribution appeared to be a logical choice. Exploratory experiments indicated that the countercurrent distribution between amyl

alcohol and water separated the leucoanthocyanin from the bulk of the other components in the ethyl acetate fraction. The amyl alcohol removed a large amount of materials from a water solution of this fraction, while the majority of the leucoanthocyanin was retained in the aqueous phase. The amyl alcohol-water system appeared to be ideal for countercurrent distribution experiments.

One gram of the ethyl acetate fraction dissolved in 20 ml. of water was extracted with 20 ml. of amyl alcohol. The upper layer was then extracted twice with water so that there were three water solutions. Each of these water solutions was extracted five times with amyl alcohol in countercurrent fashion. The yields from the water solutions were: first solution 7.9%; second solution 2.6%; third solution 1.7% giving a total of 12.2% of the starting material. Two-dimensional paper chromatograms showed only three spots for each of the water solutions, spots A, D (the leucoanthocyanin), and K. Chromatograms of the amyl alcohol solutions showed that none of the leucoanthocyanin had been extracted by the amyl alcohol.

This method offered advantages over column chromatography. A simpler mixture was obtained and no interfering substances such as dissolved Magneson were present. This method was adopted as the first step in the isolation of the leucoanthocyanin from the ethyl acetate fraction.

ISOLATION OF THE LEUCOANTHOCYANIN

The simplicity of the water-soluble fraction of the countercurrent

distribution indicated that the leucoanthocyanin could be obtained from this fraction. The first attempt at isolation was with a Magnesol column, but no separation whatever was accomplished.

Preliminary experiments indicated that neutral lead acetate when added to an aqueous solution of the water-soluble fraction obtained from the countercurrent distribution precipitated a complex from which a nearly pure leucoanthocyanin could be obtained. A saturated solution of neutral lead acetate was added to a dilute aqueous solution of the water-soluble fraction until no further precipitation was observed. The solution was centrifuged, and the resulting greenish-brown precipitate was washed several times with water, was slurried with water, and acidified with a few drops of acetic acid. Hydrogen sulfide was passed into the acidified solution until no more lead sulfide could be precipitated. The lead sulfide was removed by centrifugation and the clarified supernatant solution was extracted with ethyl acetate. A paper chromatogram of the ethyl acetate extract showed a faint spot for d-catechin and a strong spot for the leucoanthocyanin.

The latter was purified by dissolving it in a minimum of methanol and precipitating it into ethyl ether (10). The off-white precipitate was collected by centrifuging and was dried under vacuum. Paper chromatograms showed this material to be the pure leucoanthocyanin.

The yield of purified leucoanthocyanin was 0.7% based on the ethyl acetate fraction. Undoubtedly, losses occurred during the isolation and purification. The major points of loss were probably the ethyl

acetate extraction and the ether precipitation. Also, a certain amount of loss was due to the decomposition of the leucoanthocyanin. This decomposition was noted by a change in color from nearly white to dark brown. The decomposition seemed to be accompanied by a polymerization of some sort, as a chromatogram of the darkened leucoanthocyanin showed a spot for the leucoanthocyanin plus a streak following it.

CHARACTERIZATION OF THE LEUCOANTHOCYANIN

IDENTIFICATION OF CYANIDIN

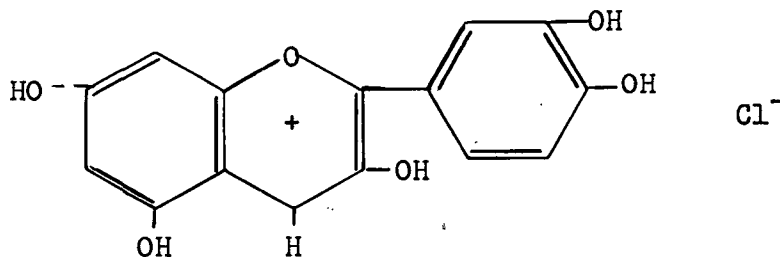
The isolated leucoanthocyanin was treated with 95% ethanol 0.4N in hydrochloric acid for forty minutes on a steam bath. The resulting red solution was concentrated to a small volume and was chromatographed. The papers were developed with the Forestall solvent and with 90% formic acid-3N HCl. The R_f values were 0.50 for the Forestall solvent and 0.22 for the 90% formic acid-3N HCl developer. These values corresponded exactly to those reported by Roux (45) for cyanidin.

The next step in the identification of cyanidin was to perform the Robinsons' (5) qualitative tests. An amyl alcohol solution of the anthocyanidin was placed over 1% hydrochloric acid and sodium acetate was added. A few drops of ferric chloride solution were added and the amyl alcohol layer turned blue indicating that cyanidin was present.

Strong confirming evidence for the identity of anthocyanidins can be obtained from spectral measurements. The wavelength of maximum absorption of most anthocyanidins falls in the 530 to 550 mμ range so

that little information can be gained from the maximum alone. However, this maximum coupled with the shift in the maximum caused by complexing the anthocyanidin with aluminum ion is quite diagnostic. Only anthocyanidins containing 3',4' vicinal hydroxyls are capable of forming this complex (53).

Since the aluminum complex of cyanidin cannot be formed in acid solution (54), quite an experimental problem was presented, inasmuch as the leucoanthocyanin must be treated with acid to form the anthocyanidin. Several attempts to neutralize the acid in the ethanol solution led to the destruction of the anthocyanidin. Finally, by keeping the ethanol solution of the anthocyanidin for several days in a vacuum desiccator over calcium oxide, its acidity was reduced to a point at which the aluminum complex could be formed. The absorption measurements were made using the solvents suggested by Geissman (54). Table V lists the wavelength of maximum absorption of the various solutions of the anthocyanidin and compares these values with those reported in the literature for cyanidin. It is clear that the values obtained compare very well with those reported for cyanidin. The spectral evidence coupled with the chromatographic evidence and the Robinsons' tests confirms the fact that the anthocyanidin is cyanidin.



Cyanidin Chloride

TABLE V

ABSORPTION MAXIMA FOR THE ANTHOCYANIDIN

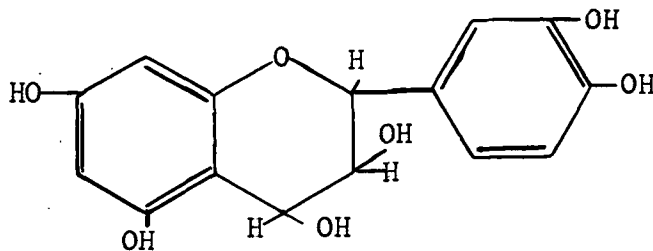
Solvent	Anthocyanidin from Leucoanthocyanin, mμ	Cyanidin, mμ
Absolute ethanol	545	546 (53)
50% ethanol	538	534 (54)
50% ethanol- AlCl_3 ^a	568	567 (54)
50% ethanol-HCl- AlCl_3 ^b	538	534 (54)

^a Two drops of 2% alcoholic AlCl_3 added to the 3-ml. spectrophotometer cell containing the solution of the anthocyanidin.

^b One drop of 0.5N alcoholic HCl and two drops of 2% alcoholic AlCl_3 added to the 3-ml. spectrophotometer cell containing the solution of the anthocyanidin.

SYNTHESIS OF THE LEUCOANTHOCYANIN

The identification of cyanidin fixed the aromatic hydroxylation pattern of the isolated leucoanthocyanin. This compound was believed to be a monomeric flavan-3,4-diol, because of its chromatographic behavior. Thus, the possibility was presented that the leucoanthocyanin was the same as Swain's (27) synthetic product. Swain (27) reduced dihydroquercetin with sodium borohydride and obtained a flavan-3,4-diol which he called "leucocyanidin". This compound yielded cyanidin upon treatment with hot dilute acid.



Leucocyanidin

The reduction was carried out as follows. A 100-mg. sample of dihydroquercetin was dissolved in 3 ml. of methanol and added to 5 ml. of 0.1N sodium hydroxide containing 0.005 g. of sodium borohydride. The reaction was allowed to continue for 1.5 hours at room temperature. The reaction mixture was then acidified and concentrated at reduced pressure until all the methanol was removed. The aqueous solution was extracted with ethyl acetate and the extract taken to dryness at reduced pressure. The residue was dissolved in methanol and precipitated in ethyl ether. It was necessary to repeat the precipitation three times to remove all the impurities. The yield of purified product was 22% of the theoretical yield. This product was used for a series of comparisons with the isolated leucoanthocyanin.

As a check on the identity of the synthetic product, it was heated in ethanol 0.4N in hydrochloric acid and then treated in the same manner as the isolated leucoanthocyanin. Paper chromatograms run in the Forestall solvent and the 90% formic acid-3N HCl developer gave R_f values corresponding to those for cyanidin. The wavelength of maximum absorption was 538 m μ and the aluminum chloride shift was to 570 m μ in agreement with the values for cyanidin.

COMPARISON OF THE SYNTHETIC AND ISOLATED LEUCOANTHOCYANINS

In order to establish the similarity between the two leucoanthocyanins, a series of comparisons were made. The two compounds were compared with respect to their chromatographic behavior, and their infrared and ultraviolet spectra. Both materials had the same physical

appearance when freshly precipitated and both turned dark brown upon standing. Paper chromatograms of the two materials yielded similar results both for the freshly precipitated material and for the aged material.

Ultraviolet Spectra

Ultraviolet spectra were determined for both compounds in purified 95% ethanol. These spectra are depicted in Figure 2. The spectra are very similar except for the 220 to 235 mμ range. Here the isolated material appears to show slightly higher absorption. It is doubtful that these differences are real, since the solvent cut-off and instrument cut-off are in this range. This means that the sensitivity of the instrument is reduced and that the measurements are subject to error.

The wavelength of maximum and minimum absorption and the absorptivities at maximum absorption are recorded in Table VI. The values reported in the literature for two other leucoanthocyanins which yield cyanidin are also recorded in this Table.

TABLE VI

ULTRAVIOLET ABSORPTION OF LEUCOANTHOCYANINS

Leucoanthocyanin	$\lambda_{\text{max.}}$ mμ	$E_{1\text{ cm.}}^{1\%}$	$\lambda_{\text{min.}}$ mμ
Isolated leucoanthocyanin	282	0.175	258
Synthetic leucoanthocyanin	282	0.181	258
Cacao leucoanthocyanin (<u>14</u>)	280	0.179	258
Pinus leucoanthocyanin (<u>14</u>)	280	0.173	260

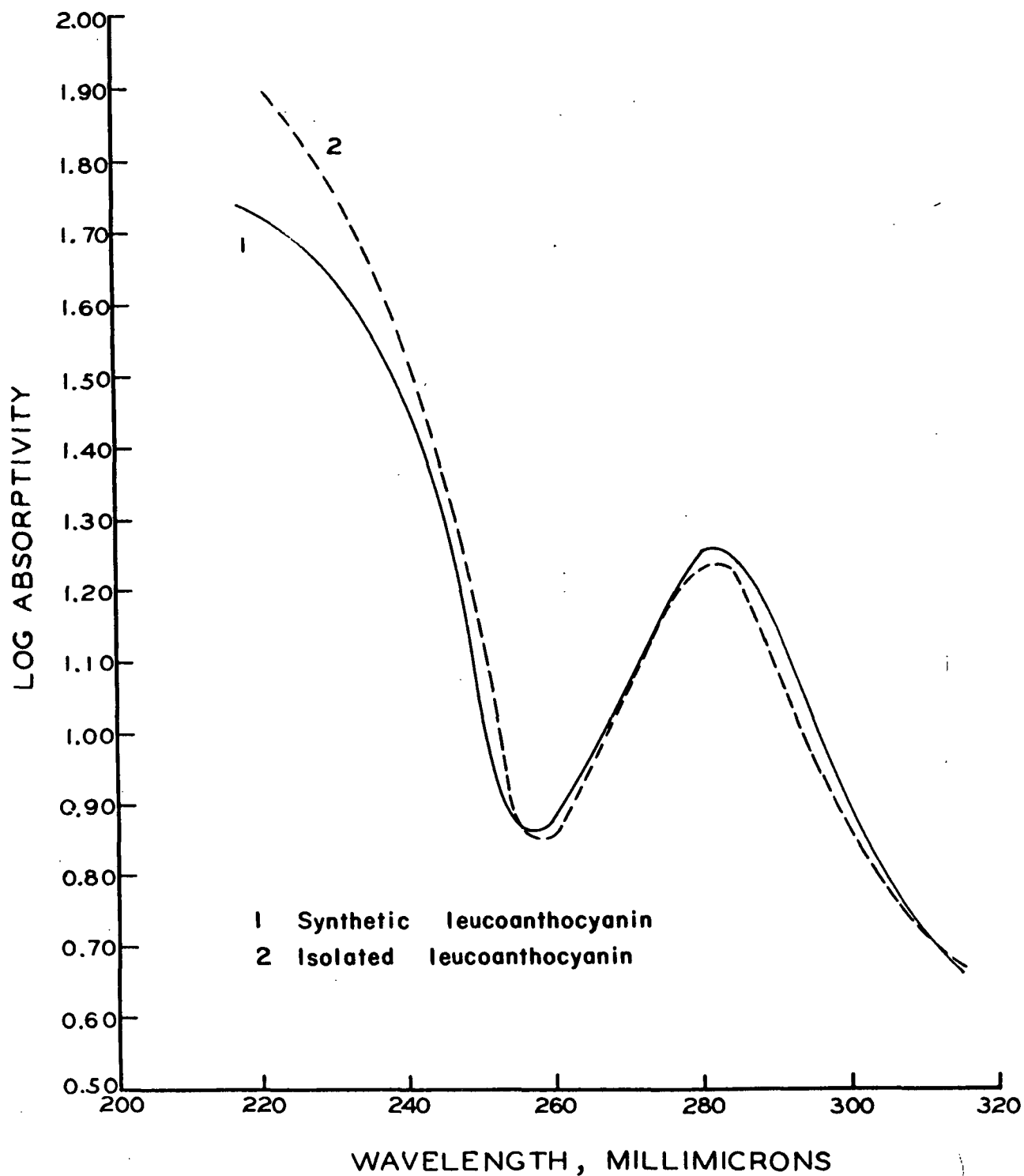


Figure 2. Ultraviolet Spectra of the Isolated and Synthetic Leucoanthocyanins

Infrared Spectra

The infrared spectra of the freshly precipitated leucoanthocyanins were identical. In the case of the synthetic product, it was necessary to make several purifications to obtain a constant spectrum. Certain absorption bands in the impure synthetic material corresponded to characteristic bands in the spectrum of dihydroquercetin, the starting material for the synthesis, as determined by Hergert and Kurth (55). Upon further purification these bands disappeared and the spectrum of the synthetic material matched that of the isolated leucoanthocyanin exactly. The spectra of these compounds are shown in Figure 3.

As would be expected, there is strong hydroxyl absorption at 3400 cm.^{-1} . The aromatic bands are present at 1620 and 1520 cm.^{-1} . It seems probable that the band at 2900 cm.^{-1} can be attributed to aliphatic CH vibrations. Hydroxyl absorption in the $1300\text{ to }1400\text{ cm.}^{-1}$ region obscures the identification of other characteristic absorption bands for these compounds. The noncrystallinity of these compounds may be responsible for the broadening of the bands in the 650 to 1000 cm.^{-1} region and the general lack of sharpness for the whole spectrum.

Summary

The isolated leucoanthocyanin is believed to be "leucocyanidin" for the following reasons: both materials yield cyanidin; they have identical infrared spectra; they give very similar ultraviolet spectra; and they exhibit the same chromatographic behavior. Due to the noncrystalline nature of both materials, melting points and mixed melting

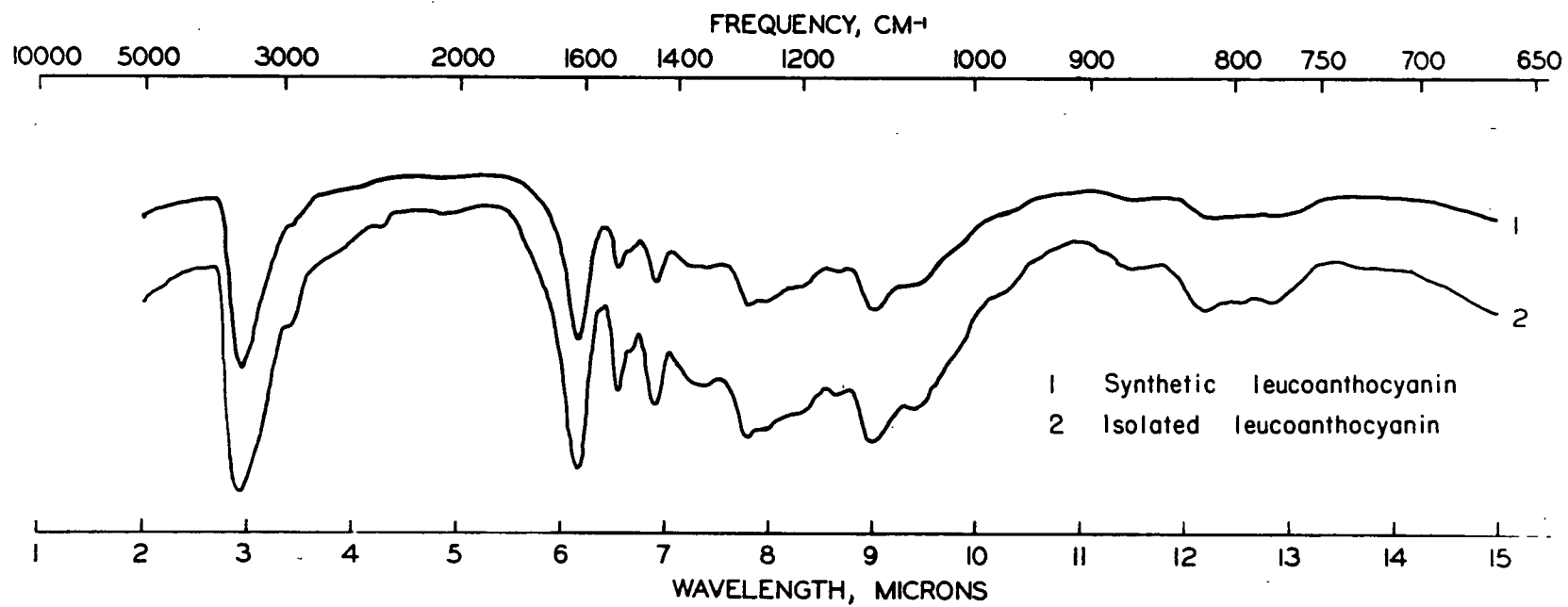


Figure 3. The Infrared Spectra of the Isolated and Synthetic Leucoanthocyanins

points could not be taken. Although this proof is not unequivocal, it is a strong indication that the isolated leucoanthocyanin is "leucocyanidin".

THE NON-LEUCOANTHOCYANIN MATERIALS

THE ACETATE

The low yield of leucoanthocyanin indicated that there were many other components in the ethyl acetate fraction, some of which were present in far greater amounts than the leucoanthocyanin. Pigman *et al.* (31) made an acetate from this fraction which erroneously was believed to be a derivative of the leucoanthocyanin.

To investigate the nature of the acetate, an acetylation was made following the procedure of Pigman. One gram of the ethyl acetate fraction was dissolved in seven milliliters of pyridine and cooled to 0°C. Fifteen milliliters of acetic anhydride, cooled to 0°C., were added and the reaction mixture was kept at 0°C. for 48 hours. At the end of this time the reaction mixture was poured into 80 ml. of ice and water. A sticky gum formed which powdered upon rubbing. The yield was 1.70 g. The crude acetate was crystallized from methanol and yielded a product with a melting point of 153-55°C. and a specific rotation of $-18.37 \pm 1.10^\circ$ in acetone. The acetate obtained by Pigman had a melting point of 153-54°C. and a rotation of -15.20° in chloroform. A mixed melting point of the acetate prepared by Pigman and the acetate obtained in the present work showed no depression. The infrared spectra of the two materials were identical. The spectra showed a band at 3000 cm.^{-1}

indicating aliphatic unsaturation. The similarity of the acetate obtained, in this work to the acetate of the stilbene glucoside obtained by Andrews (32), and the indication of aliphatic unsaturation from the infrared spectra suggested that this acetate might be a stilbene glucoside identical with that of Andrews or at least very similar.

The acetate crystallized once, was recrystallized five more times from methanol. The last crystallization yielded long needles which melted at $161-3^{\circ}\text{C}$. and had a rotation of $-21.30 \pm 1.14^{\circ}$ in acetone. Another recrystallization did not change these values. Andrews' compound had a melting point of $162-3^{\circ}\text{C}$. and a rotation of -22.6° in acetone. The similarity of the properties of these two compounds indicated that they were identical.

Deacetylation

Deacetylation was attempted using the procedure of Wolfrom and Dacons (56). An impure sample of the acetate (m.p. $153-4^{\circ}\text{C}$.), 77 mg., was dissolved in 7 ml. of 0.2N sodium methylate in methanol and stirred for one hour at 30°C . At the end of one hour the solution was acidified with acetic acid and passed through a column of Amberlite IR-120. The column was washed with 400 ml. of water and the resulting solution evaporated to a small volume at reduced pressure. A two-dimensional paper chromatogram of the deacetylated product showed spots A, B, H, and I, indicating that the acetate was impure and possibly that the deacetylation had changed the nature of the acetylated product. A check of the deacetylation procedure established that the ion-exchange resin had no

effect on the products, but that the sodium methylate solution did affect the unacetylated ethyl acetate fraction. Since Andrews had been unable to deacetylate his stilbene glucoside acetate, and since the deacetylating agent was shown to change the products, deacetylation was abandoned.

Oxidation

King et al. (57) oxidized the acetates of several stilbenes, deacetylated the oxidation products, and identified them as substituted benzoic acids. Chromic acid in acetic acid and potassium permanganate in acetone were used as the oxidants. Both of these methods were tested in the present work. A 55-mg. sample of the acetate (m.p. 158-9°C.) was dissolved in 30 ml. of glacial acetic acid. Thirty-five milligrams of chromium trioxide was added and the reaction mixture maintained at 55°C. for one hour. Consumption of the oxidant was noted by the change in color of the solution from brown-red to green. At the end of the reaction time the solution was poured into 100 ml. of water, and the water solution was extracted several times with ethyl ether. The ether solution was taken to dryness, and a few milliliters of dilute sodium hydroxide were added. This solution was warmed for 0.5 hour and then acidified with dilute sulfuric acid. The acidified solution was extracted with ethyl ether, and the ether solution concentrated to a small volume.

Paper chromatograms of the oxidation products run in TAW and BzAW revealed spots which corresponded to known vanillic and p-hydroxybenzoic acid. Chromatograms from both developers showed spots at the origin

which could not be identified. This oxidation and deacetylation procedure was repeated twice and the same results were obtained.

A very small amount of crystalline material separated from the combined concentrated ether solutions. These crystals were collected and redissolved in high boiling petroleum ether. A few small white crystals separated from solution upon cooling. The substance melted at 201-3°C. with sublimation. This material was probably vanillic acid (m.p. 207°C. with sublimation), but unfortunately enough material could not be obtained for a mixed melting point with authentic vanillic acid.

The second oxidation procedure was with potassium permanganate in acetone. A 48-mg. sample of the acetate (m.p. 158-9°C.) was dissolved in 10 ml. of acetone and 40 mg. of potassium permanganate in 20 ml. of acetone was added. The reaction was allowed to continue at room temperature for 16 hours. At the end of the reaction period a few crystals of sodium bisulfite were added to destroy the residual permanganate. The manganese dioxide was filtered off and washed with hot water. The water wash and the acetone solution were combined and evaporated at reduced pressure until all the acetone was removed. The solution was then acidified with dilute sulfuric acid and extracted with ether. The ether extract was taken to dryness and resuspended in dilute sodium hydroxide. The alkaline solution was warmed until all the material had dissolved. The solution was then acidified and extracted with ether. Chromatograms of the ether extract were run in TAW and BzAW. As before, three spots were detected, one for vanillic acid, one

for *p*-hydroxybenzoic acid, and a third spot at the origin.

The ether solution was taken to dryness and 10 ml. of 10% sulfuric acid was added. The solution was refluxed for one-half hour, cooled, and extracted with ethyl ether. A chromatogram of the ether solution in BzAW showed four spots, one for vanillic acid, a second for *p*-hydroxybenzoic acid, a third for protocatechuic acid, and a fourth for α -resorcylic acid (3,5-dihydroxybenzoic acid). The α -resorcylic acid and vanillic acid were present in much larger amounts than were the other two acids.

Chromatograms of the products from and oxidation and subsequent hydrolysis of the pure acetate (m.p. 161-3°C.) showed strong spots for vanillic acid and α -resorcylic acid and only very faint spots for the other two acids. These results indicated that the acetate was not entirely pure, and that it was probably the same as Andrews' stilbene glucoside acetate.

ANALYSIS AND INFRARED SPECTRUM

The melting point and rotation of the purified acetate coupled with the results of the oxidation experiments indicated fairly strongly that a portion of the acetate was the stilbene glucoside acetate prepared by Andrews (32). For further support for this hypothesis, a sample of the purified acetate was analyzed for carbon, hydrogen, and methoxyl. The results agreed with the values for the stilbene acetate. Table VII gives a comparison of the properties of the purified acetate with the acetate of the stilbene glucoside obtained by Andrews. These

data confirm the hypothesis that the purified acetate is Andrews' stilbene glucoside acetate.

An infrared spectrum of the stilbene acetate is depicted in Figure 4. The prominent absorption bands may be correlated with functional groups as follows. The absorption at 1755 cm.^{-1} is due to the ester carbonyl, while the absorption at 1195 to 1240 cm.^{-1} is due to phenol acetates and aliphatic hydroxyl acetates. The doublet at $1595 - 1610\text{ cm.}^{-1}$ is probably caused by a $\text{C}=\text{C}$ conjugated with the aromatic rings and aromatic absorption. The second aromatic band is at 1510 cm.^{-1} . The sharp band at 970 cm.^{-1} may be due to the glucoside portion of the molecule or more probably to the trans-ethylenic configuration. Kuhn (58) has shown that methyl β -D-glucoside tetraacetate absorbs strongly at 910 cm.^{-1} and that glucose and D-glucal 3,4,6-triacetate absorb strongly at 1370 cm.^{-1} . Therefore, the bands at 1370 and 910 cm.^{-1} are probably due to the glucoside acetate portion of the molecule.

TABLE VII

COMPARISON OF ACETATES

Property	Black Spruce Acetate	Andrews' Acetate (32)
Melting point	$161-3^{\circ}\text{C.}$	$162-3^{\circ}\text{C.}$
$[\alpha]_D^{22}$	$-21.3 \pm 1.14^{\circ}$ in acetone	-22.6° in acetone
Analysis ^a	C, 59.0%; H, 5.54%; OCH ₃ , 4.40%	C, 58.9%; H, 5.5%; OCH ₃ , 4.5%
Oxidation products	vanillic acid and α -resorcylic acid	vanillic acid and α -resorcylic acid

^a The theoretical analysis for the stilbene acetate is C, 58.9%; H, 5.36%; OCH₃, 4.62%.

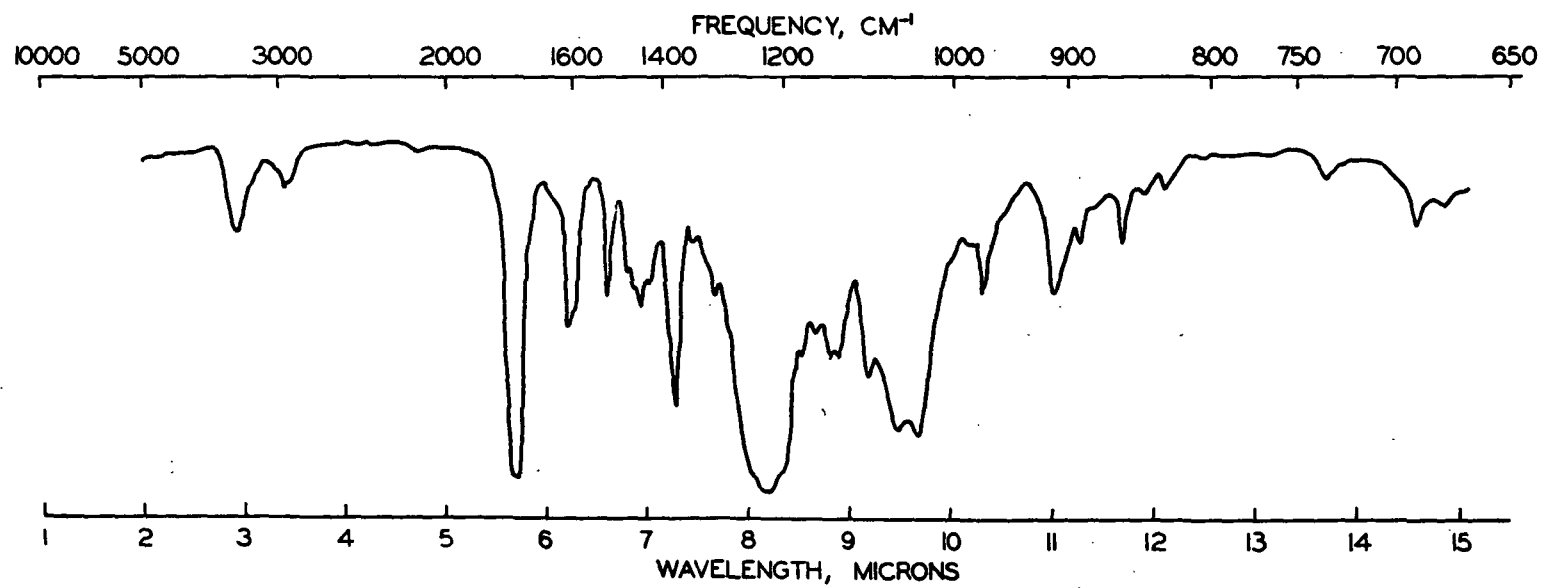


Figure 4. Infrared Spectrum of the Stilbene Glucoside Acetate

ESTIMATION OF THE STILBENE IN THE ETHYL ACETATE FRACTION

The infrared spectra of the crude acetate, the pure stilbene acetate, and the acetate crystallized once were determined. These spectra are shown in Figure 5. The aromatic band at 1510 cm.^{-1} , the acetate bands at 1755 cm.^{-1} and $1195\text{--}1240\text{ cm.}^{-1}$, and the glucoside bands at 1375 and 910 cm.^{-1} are of exactly the same intensity for the crude acetate and the pure stilbene acetate. It is apparent from the similarity of these spectra that the crude acetate probably contains a high percentage of stilbene. It is also interesting to note that the spectra for the pure and impure acetates are nearly identical. This similarity and the results of the oxidation experiments suggest that there may be another stilbene acetate present in the impure acetate which is similar to Andrews' stilbene acetate. This possibility will be discussed more fully later.

An attempt was made to quantitatively determine the amount of stilbene in the ethyl acetate fraction. It was assumed that all the stilbene was present in the crude acetate. By estimating the stilbene in the acetate, the stilbene in the ethyl acetate fraction could be determined. The acetate was oxidized with permanganate in acetone, and the products were deacetylated and hydrolyzed by the procedure previously described. The amount of substituted benzoic acids produced was determined by the method of Pearl *et al.* (59). The solution of the acids was made up to a known volume, an aliquot was streaked across the top of a sheet of paper, and the paper was developed in BzAW. The bands containing the acids were located by spraying a test strip with PNA.

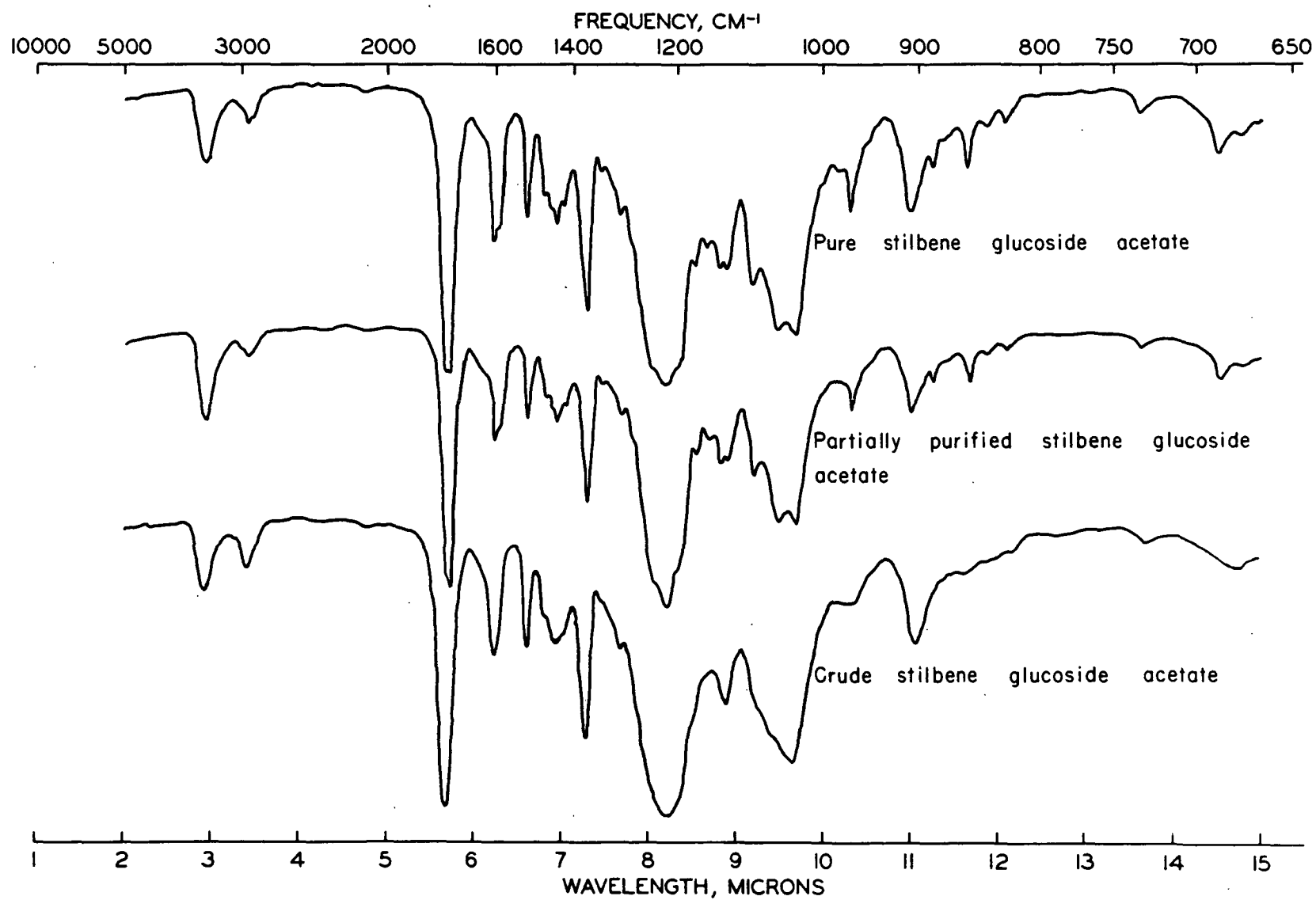


Figure 5. Infrared Spectra of the Black Spruce Acetates

Each of the bands was cut from the chromatogram and eluted with 95% ethanol. The ethanol eluates were made alkaline, diluted to a known volume, and their concentrations determined photometrically. These values were used to calculate the initial concentration of stilbene. Unfortunately, it was found that the oxidation was not quantitative and this method of estimation could not be used.

Since a quantitative estimation could not be made, a rough estimate was made by two methods. The first method was based on the amount of glucose obtained by hydrolysis of the ethyl acetate fraction. All the glucose was assumed to be associated with the stilbenes and was estimated at $20 \pm 5\%$. Since the stilbene glucosides yield 43% glucose, the amount of stilbene glucoside in the ethyl acetate fraction was calculated as $(20 \pm 5 / 43) 100$ or 35 to 58%.

The second estimation was based on the acetate. Since the infrared spectra of the pure stilbene acetate and the impure acetate crystallized once were nearly identical, it was assumed that the impure acetate represented only substituted stilbenes. The impure acetate was also assumed to represent all the substituted stilbene in the crude acetate. On acetylation, one gram of the ethyl acetate fraction yielded 1.65 g. of crude acetate of which 0.858 g. or 50% was recovered on the first recrystallization and represented the total stilbene acetate. This value was converted into its unacetylated weight. The result, 0.515 g. or 51.5% of the ethyl acetate fraction, was substituted stilbene glucoside.

The two estimated values agree fairly well and indicate that the stilbene glucoside represented 40 to 60% of the ethyl acetate fraction.

THE PROPANOL HYDROLYSIS TEST

Pigman et al. (31) devised a semiquantitative spectral method for determining leucoanthocyanins in an extract. The test results were reported as absorptivities rather than percentage or concentration of leucoanthocyanin. The test results depended upon the concentration of leucoanthocyanin. For highest accuracy, the method specified that a sample size be taken that would yield a final transmittance of 20 to 30%. The sample was dissolved in propanol, 3 ml. of 3N hydrochloric acid were added, and the solution was diluted to 10 ml. with propanol. The contents of the flask were poured into a glass tube equipped with a stopper and the stopper seated tightly. The tube was placed in a boiling water bath and heated for forty minutes. It was then removed, cooled, and the contents poured into a 1-cm. spectrophotometer cell. The absorbance at 540, 545, and, 550 mμ was measured and averaged. The absorptivity was then calculated by dividing the absorbance by the concentration in mg. per ml.

The synthetic leucoanthocyanin appeared to be a suitable material for the standardization of this test. Accordingly, a series of samples of different concentrations of synthetic leucoanthocyanin were analyzed by this method. The results recorded in Table VIII agree with the previous work in that the absorptivity of the final solution was dependent on the initial concentration.

TABLE VIII

STANDARDIZATION OF THE
PROPANOL HYDROLYSIS TEST

Concentration, mg./10 ml.	Absorbance	Absorptivity
2.84	1.560	5.51
1.12	0.859	7.65
0.56	0.495	8.91
0.28	0.307	10.79
0.12	0.133	11.60
0.06	0.080	14.35

To determine the source of concentration dependency, a calculation of the percentage conversion into anthocyanidin was made. For this calculation, the absorptivity of cyanidin given by Schou (60) was used to estimate the absorbance of a solution in which all the leucoanthocyanin had been converted into anthocyanidin. This value was compared to the actual absorbance of this solution. The conversion varied from 7 to 20% over the concentration range studied with conversion increasing with decreasing concentration. Within the specified transmittance range of 20 to 30% the variation was only 2%.

The propanol hydrolysis test was applied to the ethyl acetate fraction over a range of concentrations. The results followed the same general trend of increasing absorptivity with decreasing initial concentration as shown in Table IX. The absorptivities were much lower than those reported by Pigman et al. (31) indicating a lower leucoanthocyanin content.

To compare the results of the determinations run by Pigman et al.

with those obtained in this work," the percentage leucoanthocyanin was calculated using the synthetic "leucocyanidin" as a standard. A plot of initial concentration versus absorbance was made for the synthetic material," the Pigman et al. ethyl acetate fraction," and the ethyl acetate fraction from the present work. The concentrations of all three materials were determined at absorbances arbitrarily chosen from the plot. The concentration of synthetic "leucocyanidin" was assumed to represent the concentration of leucoanthocyanin in the two ethyl acetate fractions. The percentage leucoanthocyanin was then calculated by dividing the concentration of synthetic "leucocyanidin" by the appropriate concentration of the ethyl acetate fraction and multiplying by 100. Table X records the absorbance chosen and the percent leucoanthocyanin calculated for the two ethyl acetate fractions at these absorbances.

TABLE IX

PROPANOL HYDROLYSIS TEST ON THE
ETHYL ACETATE FRACTION

Concentration, mg./10 ml.	Absorbance	Absorptivity
4.55	0.412	0.91
2.75	0.305	1.10
2.00	0.204	1.02
1.44	0.170	1.18
0.60	0.064	1.07
0.36	0.045	1.25

The data indicated that using the synthetic "leucocyanidin" as a standard extended the transmittance range in which consistent results are obtained beyond that originally specified. They also indicated that the ethyl acetate fraction prepared by Pigman et al. was

richer in leucoanthocyanin than the one obtained in the present work. This may be due to many factors. Collection during different seasons could have caused this great difference. It has been shown that in some plants the seasonal variation of the concentration of the leucoanthocyanin is quite large (61). Other contributing factors may be variation in the isolation technique, different geographic location of the trees sampled, trees of different age, and a normal tree to tree variation.

TABLE X

ESTIMATION OF THE LEUCOANTHOCYANIN BY
THE PROPANOL HYDROLYSIS TEST

Transmittance, %	Absorbance	Leucoanthocyanin, % Pigman <i>et al.</i> This work ^a	
20	0.70	31.6	—
30	0.52	30.4	—
40	0.40	30.6	9.9
50	0.30	30.6	10.2
60	0.22	30.4	11.2
70	0.15	31.8	10.7
80	0.10	30.8	9.8

^a The sample had aged for nine months before testing so that these values may be low due to the decomposition of the leucoanthocyanin.

The values given by the test may be somewhat high due to extraneous materials absorbing in the 540 to 550-m μ range. A correction can be made for the materials which absorb in this region before acid treatment. In the present experiments the absorption of the original solutions was negligible. However, the materials which absorb after acid treatment produce an error which cannot be corrected.

The ethyl acetate fraction obtained in the present work yielded a value of 10% leucoanthocyanin. Although its actual concentration is not known, isolation experiments, i.e., countercurrent distribution and column chromatography, indicated that the leucoanthocyanin was less than 10% of this fraction. In this case the interfering materials evidently contributed little error to the measurement.

The use of a standard increased the usefulness of the propanol hydrolysis test by increasing the concentration range in which consistent results were obtained and by allowing the concentration of the leucoanthocyanin to be calculated. However, this standard may not be applicable for all leucoanthocyanin determinations. If the conversion of other leucoanthocyanins is higher than in the present case, erroneous results will be obtained using this standard. Also, the leucoanthocyanins which produce anthocyanidins other than cyanidin will require a different standard. Therefore, caution should be exercised in using this standard for the calculation of the concentration of leucoanthocyanins in other extracts.

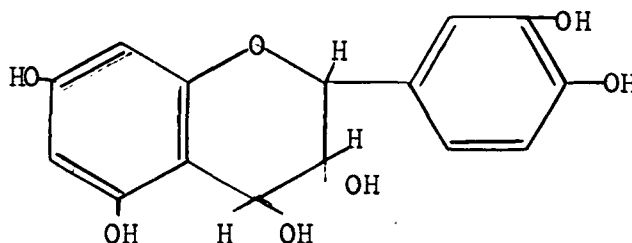
DISCUSSION

THE LEUCOANTHOCYANIN

The yield of isolated leucoanthocyanin represented 0.7% of the fraction recovered by ethyl acetate. Presumably this is a minimal figure, since the losses during the isolation undoubtedly reduced the yield. Also, there was probably additional loss due to the decomposition of the leucoanthocyanin. Apparently, the isolated leucoanthocyanin was unstable, since within a few days it became discolored and its solubility decreased. Due to this instability, an accurate estimation of the amount of leucoanthocyanin originally present in this fraction is quite difficult. Isolation experiments, i.e., column chromatography and countercurrent distribution, indicated that the leucoanthocyanin constituted less than 10% of the ethyl acetate fraction. The propanol hydrolysis test described previously yielded a value of 10% leucoanthocyanin, which is probably somewhat high, but which gives the order of magnitude. Thus, the leucoanthocyanin comprises approximately 10% of the ethyl acetate fraction.

The structure of the leucoanthocyanin has been quite well established as 3',4',5,7-tetrahydroxy-flavan-3,4-diol. This structure harmonizes with the current concept of the leucoanthocyanins, since the flavan-3,4-diol structure has been accepted generally as one of the basic structural types for the leucoanthocyanins (15 - 19). Treatment of this compound with hot dilute acid produced the expected products. A chromatogram of these reaction products showed a spot for

cyanidin and a trailing smear for the "phlobaphene" material. Catechin was not in evidence. These findings coincide with the data obtained by Swain (27) for his synthetic "leucocyanidin". They also agree with the results obtained by King and Bottomley (15), although their anthocyanidin differed from that given above because of a different hydroxylation pattern.



3',4',5,7-Tetrahydroxy-flavan-3,4-diol

The reason some leucoanthocyanins produce catechin whereas others do not, is not known at present. As mentioned previously, Forsyth (10) has shown that one of the leucoanthocyanins isolated from cacao beans produced rather large amounts of catechin. The structure of this compound remains undetermined. Presumably, this compound has certain structural differences from the leucoanthocyanin isolated in the present work. There must also be certain similarities between these two compounds as they both yield cyanidin and give similar ultraviolet spectra. With compounds of this type the differences in the nonaromatic portion of the molecule often have little or no effect on their ultraviolet spectra (27, 61), and if the two compounds have the same aromatic substitution patterns and only slightly different nonaromatic portions, they could give the same ultraviolet spectra. Since the structure of Forsyth's leucoanthocyanin is not known, the differences between the two

compounds cannot at present be attributed only to the nonaromatic portion of the molecules.

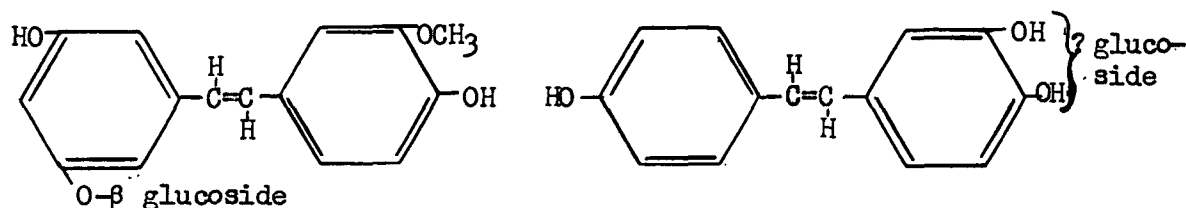
The proof of structure of the isolated leucoanthocyanin adds further support to the concept that flavan-3,4-diols represent a basic structural type for the leucoanthocyanins. However, it does not aid in explaining the different chemical behavior of other leucoanthocyanins. Neither does it contribute to the understanding of the complex leucoanthocyanins referred to in an earlier part of this thesis. Much further work will be necessary before the leucoanthocyanins are understood completely.

THE STILBENES

During the isolation and characterization of Andrews' stilbene glucoside in the present work, data were obtained which indicated the presence of a possible second stilbene glucoside. This was first indicated in the oxidation experiments. Oxidation of the acetate, which had been crystallized only once, produced four substituted benzoic acids; vanillic acid, p-hydroxybenzoic acid, α -resorcylic acid and protocatechuic acid. The vanillic acid and the α -resorcylic acid were derived from Andrews' stilbene; the source of the p-hydroxybenzoic acid and the protocatechuic acid was unknown. Since these two acids could not be detected in the original ethyl acetate fraction, they probably resulted from some compound with a linkage susceptible to oxidation under the same conditions as those used in the oxidation of Andrews' stilbene. These two acids were present in about the same amounts, but in considerably smaller quantity than were the vanillic and α -resorcylic acids.

Since the protocathechuic acid could not be detected among the oxidation products until after they had been hydrolyzed with acid, it appears that one of the hydroxyls of the protocathechuic acid was combined in some manner, probably as a glycoside. The sugar residue was doubtless glucose, as it was the only one detectable among the hydrolysis products mentioned previously.

The hypothesis that the protocathechuic acid portion of the unknown compound is glycosylated was given support by chromatograms of the ethyl acetate fraction which showed only ferric chloride positive spots for d-catechin and the leucoanthocyanin. Had the vicinal phenolic hydroxyls of the protocathechuic acid been free, they also should have reacted with the ferric chloride spray. The possible structure of the hypothesized second stilbene glucoside is given below, together with that of Andrews' stilbene glucoside.



Andrews' Stilbene Glucoside

Proposed Partial Structure for a Possible Second Stilbene Glucoside

Another indication of the presence of a second stilbene glucoside was given by the infrared spectra of the acetates (Figure 5). The spectrum of the acetate crystallized only once is identical with that of Andrew's stilbene glucoside acetate. If this crystallized acetate contained appreciable amounts of compounds with structures different

from that of the stilbenes, its spectrum would certainly show absorption bands different from those in the spectrum of Andrews' stilbene acetate. This was not the case. Presumably, the two stilbenes should show different infrared spectra. The differences should be most noticeable in the regions correlated with aromatic substitution (1400 to 1600 cm.^{-1} and 700 to 900 cm.^{-1}). However, with a high degree of acetylation the absorption due to the acetyl groups could blot out such differences. Also, the absorption due to the glucose and the acetyls would tend to dilute the aromatic substitution bands to a point at which they were not distinguishable.

The presence of this second stilbene remains hypothetical and at present cannot be supported by unequivocal experimental evidence, so it is proposed with reservations. The detection of protocathechuic acid and *p*-hydroxybenzoic acid among the oxidation products simply indicates that another compound must be present in the initially crystallized acetate, but does not prove that it is a stilbene. The same can be said for the inferences drawn from the infrared spectra. To actually establish the presence of the second stilbene would require its isolation and characterization; the problem of isolation and purification would be exceedingly difficult, since this compound is probably present in much smaller amounts than is Andrews' stilbene and because of its structural similarity to Andrews' compound.

RE-EVALUATION OF THE DATA OF PIGMAN et al.

During the investigation of the color precursors of black spruce inner bark, Pigman et al. (31) prepared an ethyl acetate fraction very

similar to the one obtained in this work. They referred to this fraction as the "black spruce inner bark leucoanthocyanin". They believed that this fraction was mostly leucoanthocyanin and subjected it to analysis. Hydrolysis studies indicated the presence of a glucoside. Functional group analysis indicated the presence of a methoxyl group. They also prepared a crystalline acetate which they believed to be a derivative of the leucoanthocyanin. In addition they methylated the so-called "leucoanthocyanin" and obtained two products differing in methoxyl contents. Analysis of all the products indicated to them that the original material was a substituted chalcone with one methoxyl, two phenolic hydroxyls, two alcoholic hydroxyls, and a glucose residue.

The data collected in the present work clearly indicated that a reinterpretation of the data obtained by Pigman et al. was necessary. The leucoanthocyanin has been shown to be but a relatively small part of the ethyl acetate fraction and furthermore that it contains neither a methoxyl nor a glucose residue. In view of the evidence collected, it seems probable that the data of Pigman et al. (31) are more in harmony with the structure of the stilbene glucosides rather than with that of a leucoanthocyanin.

The ethyl acetate fraction irrespective of whether isolated by Pigman or in the present work has properties similar to Andrews' stilbene glucoside. Both materials were light yellow in color and reduced hot Fehling solution only slightly until after hydrolysis whereupon a strong reduction was noted.

The elemental and functional group analyses of the Pigman acetate were almost the same as those for Andrews' stilbene glucoside acetate except that the methoxyl value was low. The analysis of the hypothetical second stilbene glucoside is almost the same as Andrews' stilbene glucoside with the exception of the methoxyl. A mixture of these two stilbene acetates in which Andrews' compound predominated would give the same analysis as did Pigman's acetate. It seems possible that the Pigman acetate may have been a mixture of two stilbenes. It certainly was not a derivative of the leucoanthocyanin.

Methylation of Pigman's ethyl acetate fraction yielded two products, one with a 14.6% methoxyl and a much larger amount of material with a methoxyl of 20.2% (31). These products could well have been methylated stilbenes. Andrews' stilbene when fully methylated would have a methoxyl of 20.7% which agrees reasonably well with the 20.2% determined by Pigman. The second methylated product could have come from two sources, Andrews' compound partially methylated containing 14.3% methoxyl or the second hypothetical stilbene fully methylated containing 14.8% methoxyl. To prove this hypothesis the products would have to be more fully characterized and compared with authentic samples of the methylated stilbenes.

Additional support for the idea that Pigman et al. were actually characterizing the stilbenes stems from the fact that the stilbenes represent such a large portion of the ethyl acetate fraction. Since these compounds could represent as much as two-thirds of the fraction, it is probable that any analytical or physical data on this fraction

would actually characterize these compounds, and not the leucoanthocyanin.

The isolation of a second stilbene and a more complete characterization of the methylated products would aid greatly in more fully determining the nature of the so-called "black spruce inner bark leucoanthocyanin". There are of course other components in the ethyl acetate fraction, but most of these are present in such small quantities that they contribute but little to the character of this fraction.

SUMMARY

Fresh samples of black spruce inner bark were extracted and the extract fractionated by a procedure similar to that of Pigman et al. (31). The undried bark was immediately immersed in methanol and allowed to soak at room temperature for two weeks. Subsequently the methanol was poured off, water was added, and the mixture evaporated until the methanol was removed. The residual solids were fractionated as follows. The water solution was saturated with sodium chloride, and the saturated salt solution subsequently extracted with ethyl acetate. The materials recovered by this extraction, referred to as the "black spruce inner bark leucoanthocyanin" by Pigman and termed the "ethyl acetate fraction" in the present work, served as the starting material.

Paper chromatographic examination of the materials recovered from the ethyl acetate extraction revealed that this fraction was actually a complex mixture. The chromatograms also indicated that the leucoanthocyanin constituted a relatively small portion of the materials in this extract.

Acid hydrolysis of the ethyl acetate fraction yielded 20% glucose determined chromatographically rather than the 40% reported by Pigman. The Munson-Walker method of determining reducing sugars used by Pigman is sensitive to all reducing materials, and probably some of the phenolic constituents in the hydrolyzate reacted with the Munson-Walker reagent thus producing a high result.

Attempted isolations of the leucoanthocyanin through column chromatography and countercurrent distribution did not yield a pure product, but did indicate that the leucoanthocyanin represented not over 10% of the ethyl acetate fraction.

Finally, the leucoanthocyanin was isolated by precipitation from an aqueous solution of the water-soluble materials from the countercurrent distribution with neutral lead acetate. The yield of purified leucoanthocyanin was only 0.7% of the material recovered by ethyl acetate extraction. This figure is lower than the estimated amount of leucoanthocyanin due to the losses incurred during the isolation.

Treatment of the purified leucoanthocyanin with ethanol 0.4N in hydrochloric acid produced a red-colored solution. The material producing this red color was identified as cyanidin by paper chromatography and spectral measurement of the wavelength of maximum absorption of a solution of cyanidin and the shift in this maximum caused by complexing the cyanidin with aluminum chloride.

A leucoanthocyanin, 3',4',5,7-tetrahydroxy-flavan-3,4-diol, was synthesized by reducing dihydroquercetin with alkaline sodium borohydride according to the procedure indicated by Swain (27). This compound was used to establish the identity of the isolated leucoanthocyanin through a series of comparisons of their respective physical and chemical properties. Both materials exhibited the same chromatographic behavior and both compounds produced cyanidin upon acid treatment. The ultraviolet spectra of the synthetic and isolated leucoanthocyanins

showed the same wavelengths of maximum and minimum absorption and nearly the same absorptivities. The infrared spectra of the two compounds were identical.

The materials recovered from the original ethyl acetate fraction were acetylated by the procedure given by Pigman *et al.* (31). The acetylated product when recrystallized once was identical with the acetate obtained by Pigman. Five further recrystallizations from methanol yielded a pure compound which was identified as the hexaacetate of 3,4'-dihydroxy-3'-methoxy-stilbene-5-O- β -glucoside.

The propanol hydrolysis test devised by Pigman *et al.* (31) for the semiquantitative estimation of leucoanthocyanins was evaluated using the synthetic leucoanthocyanin as a standard. Previously, only the absorptivities of the test solutions were reported and no authentic sample of leucoanthocyanin was available; therefore, the concentration of the leucoanthocyanin could not be calculated. The use of the standard extended the concentration range over which the test could furnish consistent results and allowed the calculation of the percentage leucoanthocyanin.

CONCLUSIONS

From the results of this study the following conclusions may be drawn:

1. The ethyl acetate fraction obtained in this work and previously referred to as the "black spruce inner bark leucoanthocyanin" by Pigman contains about 10% true leucoanthocyanin.
2. This fraction contains between 40 and 60% 3,4'-dihydroxy-3'-methoxy-stilbene-5-O- β -glucoside.
3. Chromatographic evidence indicates that d-catechin is present in the ethyl acetate fraction in an amount approximately equal to that of the leucoanthocyanin.
4. In addition to these compounds there are very small amounts of twelve (or more) phenolic compounds of unknown character which reacted with the diazotized p-nitroaniline spray.
5. The crystalline acetate prepared by Pigman et al. which they believed to be a derivative of the leucoanthocyanin is actually an impure preparation of the hexaacetate of 3,4'-dihydroxy-3'-methoxy-stilbene-5-O- β -glucoside.
6. The true leucoanthocyanin is not a glucoside as originally proposed and does not contain a methoxyl group.
7. The true leucoanthocyanin produces cyanidin when treated with an alcohol containing hydrochloric acid.
8. Comparison with synthetic "leucocyanidin" has established the structure of the isolated leucoanthocyanin as 3',4',5,7-tetrahydroxy-flavan-3,4-diol.

9. The semiquantitative propanol hydrolysis test devised by Pigman et al. was extended by using a suitable standard. The improved test yielded a concentration of leucoanthocyanin consistent with other experimental data, i.e., about 10% of the ethyl acetate fraction.

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